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(54) Title: SINGLE-VIAL FORMULATIONS OF DNA/LIPID COMPLEXES

(57) Abstract

The invention relates to single-vial formulations of plasmid DNA/cationic lipid complexes for human clinical use prepared by various different processes, for example, by the process comprising the steps of: (a) autoclave sterilizing a cationic lipid solution at a concentration sufficiently high to substantially prevent lipid degradation during said autoclave sterilization; (b) diluting the sterilized cationic lipid solution of step (a) to a degree sufficient to substantially prevent lipid aggregation during step (d) below; (c) filter sterilizing a plasmid DNA solution; (d) adding the sterilized plasmid DNA solution of step (c) to the diluted sterilized cationic lipid solution of step (b) at an ionic strength that is lower than isotonicity to form DNA/lipid complexes; and (e) adjusting the DNA/lipid complexes of step (d) to near isotonicity.

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SINGLE-VIAL FORMULATIONS OF DNA/LIPID COMPLEXES

FIELD OF THE INVENTION

The invention relates to single-vial formulations of plasmid DNA/cationic lipid complexes for human clinical use, and related processes.

BACKGROUND OF THE INVENTION

The use of plasmid DNA/cationic lipid complexes to transfer genes *in vivo* for the treatment of human diseases, including malignancy and cardiovascular disorders, is underway in active human clinical trials.

The Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH) approved Nabel et al. to conduct a human clinical protocol using lipid mediated transfer of an immunogen encoding gene into tumors for immunotherapy of neoplasia. Nabel et al., Proc. Natl. Acad. Sci. USA 90:11307 (1993); see Nabel et al., Human Gene Therapy 3:399 (1992); see also Nabel et al., Human Gene Therapy 5:57 (1994); PCT Patent Application WO# 94/29469. The gene encoding a foreign major histocompatibility complex protein, HLA-B7, was introduced into HLA-B7-negative patients with advanced melanoma by injection of DNA/liposome complexes using a DC-Chol/DOPE cationic lipid mixture. Nabel et al., Proc. Natl. Acad. Sci. USA, supra. Six administrations were completed without complications in five HLA-B7-negative patients with stage IV melanoma (i.e., one patient received a second administration). Plasmid DNA was detected by polymerase chain reaction within biopsies of treated tumor nodules 3-7 days after injection but was not found in the serum at any time. Recombinant HLA-B7 protein was demonstrated by immunohistochemistry in tumor biopsy tissue in all five patients, and immune responses to HLA-B7 and autologous tumors could be detected. No antibodies to DNA were detected in any patient. One patient demonstrated regression of injected nodules on two independent treatments, which was accompanied by regression at distant sites. These studies demonstrated the feasibility, safety, and therapeutic potential of lipid-mediated gene transfer in humans. See also Stewart et al., Human Gene Therapy, 3:267 (1992) (safety and nontoxicity of gene transfer *in vivo* with DNA/lipid complexes); Nabel et al., Human Gene Therapy, 3:649 (1992) (lack of autoimmunity and gonadal localization by gene transfer *in vivo* with DNA/lipid complexes); San et al., Human Gene Therapy, 4:781 (1993) (safety and nontoxicity of new cationic lipid mixture, DMRIE/DOPE, for human gene therapy).

As a result of the Nabel et al. study, Vical Incorporated proposed a multi-center clinical trial using an improved cationic lipid mixture, DMRIE/DOPE. Vogelzang et al., Human Gene Therapy 5:1357 (1994); Hersh et al., Human Gene Therapy 5:1371 (1994); and Rubin et al., Human Gene Therapy 5:1385 (1994). The Food and Drug Administration (FDA) has authorized these clinical protocols.

The FDA also recently allowed Vical Incorporated to conduct a clinical protocol using lipid mediated transfer of a cytokine encoding gene into tumors for treatment of malignancy. See Example 8. In preclinical studies, the intratumor injection of a plasmid DNA expression vector containing the human interleukin-2 (IL-2) gene reduced the incidence of tumor formation and slowed tumor growth. By local expression of cytokines at the site of the tumor, it is envisioned that lower levels of cytokines will be required for efficacy as compared to systemic administration and that these levels will be sufficiently low to avoid producing toxicity

in the patient. The findings suggest that introduction of IL-2 into a tumor, by intratumor injection of plasmid DNA expression vectors, can stimulate an antitumor response. In the proposed trial, this approach will be applied to human patients with solid malignant tumors, using a plasmid DNA that encodes the human IL-2 protein and a DMRIE/DOPE cationic lipid mixture.

The widespread utility of a polynucleotide/lipid approach has been established from studies showing cationic lipid-dependent delivery of DNA (Felgner et al., Proc. Natl Acad. Sci. U.S.A., 84:7413 (1987); Felgner, P.L., Adv. Drug Delivery Rev., 5:167 (1990); Felgner et al., Nature, 337:387 (1989); Brigham et al., Am. J. Respir. Cell. Mol. Biol., 1:95 (1989); Muller et al., DNA Cell Biol., 9:221 (1990); Burger et al., Proc. Natl. Acad. Sci. U.S.A., 89:2145 (1992)), mRNA (Weiss et al., J. Virol., 63:5310 (1989); Malone et al., Proc. Natl. Acad. Sci. U.S.A., 86:6077 (1989)), and antisense oligomers (Chiang et al., J. Biol. Chem., 266:18162 (1991); Bennett et al., Mol. Pharmacol., 41:1023 (1992)) into living cells. Since the initial published description in 1987, several reagents have become commercially available (Behr et al., Proc. Natl. Acad. Sci. U.S.A., 86:6982 (1989); Rose et al., Biotechniques, 10:520 (1991); Leventis et al., Biochim Biophys. Acta., 1023:124 (1990)), and additional cationic lipid reagents have been described reporting advantages relative to the commercial products (Farhood et al., Biochim Biophys. Acta., 1111:239 (1992); Gao et al., Biochem Biophys. Res. Commun., 179:280 (1991); Legendre et al., Pharmaceutical Res., 9:1235 (1992); Zhou et al., Biochim. Biophys. Acta., 1065:8 (1991); Pinnaduwage et al., Biochim. Biophys. Acta., 986:33 (1989)). The broad applicability of this approach has been further established in preclinical *in vivo* studies showing cationic lipid-dependent gene delivery to catheterized blood vessels (Nabel et al., Proc. Natl. Acad. Sci. U.S.A., 89:5157 (1992); Lim et al., Circulation, 83:2007 (1991); Yao et al., Proc. Natl. Acad. Sci. U.S.A., 88:8101 (1991); Nabel et al., Science, 249:1285 (1990)), lung epithelial cells (Stribling et al., Proc. Natl. Acad. Sci. U.S.A., 89:11277 (1992); Brigham et al., Am. J. Med. Sci., 298:278 (1989); Yoshimura et al., Nucleic Acids Res., 20:3283 (1992)), brain tissue (Jiao et al., Exp. Neurol., 115:400 (1992); Ono et al., Neurosci. Lett., 117:259 (1990)), Xenopus embryos (Demeneix et al., Int. J. Dev. Biol., 35:481 (1991); Holt et al., Neuron, 4:203 (1990)), and the systemic circulation (Zhu et al., Science, 261:209 (1993); Philip et al., J. Biol. Chem., 268:16087 (1993)).

Yet, formulation of plasmid DNA for therapeutic purposes requires that a pharmaceutically acceptable vehicle be found in which the DNA can be taken from the manufacturing site to the clinical site with a commercially viable interim shelf-life. Although buffers containing tris-(hydroxymethyl)aminomethane (Tris), usually incorporating chelating agents, are commonly used to handle plasmid DNA in the research laboratory, these buffers are unauthorized for parenteral use in the clinic. In their absence, a vehicle must be identified that provides for efficient handling of the plasmid DNA in the manufacturing setting, preserves the chemical and biological integrity of the plasmid DNA during shipping and storing, and allows efficient delivery of the DNA to the desired tissue target by the preferred route of administration.

The inclusion of cationic lipids in a formulation with plasmid DNA introduces additional complexity into the choice of a suitable vehicle for pharmaceutical use. In this case, the solubility and stability of the

individual oppositely charged components and the complexes they form must be accommodated in a single medium. If formation and preservation of the plasmid DNA/cationic lipid complexes is considered to be essential for the functioning of the product, it is imperative that the vehicle chosen for pharmaceutical use not interfere with this interaction.

The stability of the product can be enhanced by frozen storage, which imposes additional restrictions on the choice of vehicle for mixtures of plasmid DNA and cationic lipid. In this instance, the stability of the complexes formed between plasmid DNA and lipid must be considered in addition to that of the individual components. Specifically, the formulation must be designed so as to preserve the solubility and integrity of the plasmid DNA/lipid complexes over the course of storage at the frozen temperature.

The Nabel et al. initial work in human patients demonstrated a requirement for administration of a combination of plasmid DNA with cationic lipids for successful transfection of tumor cells. Supra. Indeed, many *in vivo* gene therapy applications have shown a requirement for administration of a mixture of DNA and lipid for efficacy. In these protocols, the gene therapy dose has been provided to the clinical site in a multiple vial configuration because the plasmid DNA/cationic lipid complexes are not stable. In this configuration, the plasmid DNA in vehicle is present in one vial. The lipid mixture is present in a another, separate vial either as a dried film or in solution. When the lipid mixture is provided in dried form, still another vial containing the diluent for rehydration of the lipid is provided to the clinic.

With this configuration of product, the clinic staff must prepare the patient dose at the site by performing several sequential dilution and addition steps. First, the clinic staff must obtain the DNA in vehicle. Second, they must obtain the lipid. Third, if it is a dried film, which event is likely because of stability issues, they must rehydrate the lipid with a formulation buffer by vortexing until homogeneous. Fourth, they must transfer the reconstituted lipid into the DNA solution and mix. Finally, fifth, they must administer the amount to the patient. Optionally, the clinic staff may prepare dilutions of DNA/lipid complexes for the administration of escalating doses.

Although this type of configuration is acceptable for Phase I clinical trials, it is cumbersome to manufacture, difficult to manage in conformity with regulatory guidelines, costly to ship and store, and complicated for clinic staff and physicians to use. Importantly, there is no control or assurance of consistency in preparation of the final product, which is the dose administered to the patient. These limitations clearly demonstrate the need for a plasmid DNA/cationic lipid single-vial formulation for human clinical use.

Accordingly, it is an object of the invention to provide a single-vial formulation in which DNA and lipid are combined in a pharmaceutically acceptable vehicle.

It is another object to provide a single-vial formulation that retains the potency of the DNA and lipid.

It is a further object to provide a single-vial formulation that enjoys stability over a time course of storage.

It is still another object to provide a single-vial formulation that is universal in application.

It is yet another object to provide a single-vial formulation that is safe as shown by suitable safety studies.

It is an additional object to provide a single-vial formulation that is easy for a clinic staff to prepare and a physician to administer.

These and other objects of the invention will be apparent to the ordinary artisan upon consideration of the specification as a whole.

SUMMARY OF THE INVENTION

The invention provides a process for making a single-vial formulation of polynucleotide/lipid complexes in a pharmaceutically acceptable vehicle for human clinical use *in vivo* or *ex vivo* comprising the steps of: (a) sterilizing a lipid solution; (b) sterilizing a polynucleotide solution; (c) combining the sterilized polynucleotide solution of step (b) with the sterilized lipid solution of step (a), in dilute form, at an ionic strength that is lower than isotonicity, to form polynucleotide/lipid complexes; and (d) adjusting the polynucleotide/lipid complexes of step (c) to near isotonicity.

The invention further provides a process for making a single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use comprising the steps of: (a) autoclave sterilizing a cationic lipid solution at high concentration; (b) diluting the sterilized cationic lipid solution of step (a); (c) filter sterilizing a plasmid DNA solution; (d) adding the sterilized plasmid DNA solution of step (c) to the diluted sterilized cationic lipid solution of step (b) at an ionic strength that is lower than isotonicity to form DNA/lipid complexes; and (e) adjusting the DNA/lipid complexes of step (d) to near isotonicity.

The invention also provides a process for making a single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use comprising the steps of: (a) autoclave sterilizing a cationic lipid solution at a concentration sufficiently high to substantially prevent lipid degradation during the autoclave sterilization; (b) diluting the sterilized cationic lipid solution of step (a) to a degree sufficient to substantially prevent lipid aggregation during step (d) below; (c) filter sterilizing a plasmid DNA solution; (d) adding the sterilized plasmid DNA solution of step (c) to the diluted sterilized cationic lipid solution of step (b) at an ionic strength that is lower than isotonicity to form DNA/lipid complexes; and (e) adjusting the DNA/lipid complexes of step (d) to near isotonicity.

The invention moreover provides a process for making a single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use comprising the steps of: (a) autoclave sterilizing a cationic lipid solution having a concentration in the range of from about 0.5 to about 5.0 M; (b) diluting the sterilized cationic lipid solution of step (a) with a diluent to achieve a concentration in the range of from about 0.01 to about 1.0 M; (c) filter sterilizing a plasmid DNA solution; (d) adding the sterilized plasmid DNA solution of step (c), having a concentration in the range of from about 0.05 to about 10 mg/mL, to the diluted sterilized cationic lipid solution of step (b) at an ionic strength that is lower than isotonicity to form DNA/lipid complexes; and (e) adjusting the DNA/lipid complexes of step (d) to near isotonicity.

The invention additionally provides a process for making a single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use comprising the steps of: (a) autoclave sterilizing a cationic lipid solution of DMRIE/DOPE, having a molar ratio in the range of from about 90:10 to about 10:90, and having a concentration in the range of from about 2 to about 10 mg DMRIE/mL; (b) diluting the sterilized cationic lipid solution of step (a) with a diluent to achieve a concentration of $\leq$ about 2 mg DMRIE/mL; (c) filter sterilizing a plasmid DNA solution; (d) adding the sterilized plasmid DNA solution of step (c), having a concentration of $\leq$ about 10 mg plasmid DNA/mL, to the diluted sterilized cationic lipid solution of step (b) at an ionic strength that is lower than isotonicity to form DNA/lipid complexes at a mass ratio of from about 50:1 to about 1:10 DNA to DMRIE; and (e) adjusting the DNA/lipid complexes of step (d) to near isotonicity with sodium chloride.

The invention furthermore provides a process for making a single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use comprising the steps of: (a) autoclave sterilizing a cationic lipid solution of DMRIE/DOPE, having a molar ratio of about 50:50, and having a concentration of about 8 mg DMRIE/mL; (b) diluting the sterilized cationic lipid solution of step (a) with a diluent to achieve a concentration of $\leq$ about 1 mg DMRIE/mL; (c) filter sterilizing a plasmid DNA solution; (d) adding the sterilized plasmid DNA solution of step (c), having a concentration of $\leq$ about 5 mg plasmid DNA/mL, to the diluted sterilized cationic lipid solution of step (b) at an ionic strength that is lower than isotonicity to form DNA/lipid complexes at a mass ratio of about 5:1 DNA to DMRIE; and (e) adjusting the DNA/lipid complexes of step (d) to near physiological salinity.

The invention, as well, provides a process for making a single-vial formulation of plasmid DNA/cationic lipid complexes in about 0.9% sodium chloride with about 1% glycerol and about 0.01% Vitamin E for human clinical use comprising the steps of: (a) autoclave sterilizing a cationic lipid solution of DMRIE/DOPE, having a molar ratio of about 50:50 molar ratio, and having a concentration of about 8 mg DMRIE/mL; (b) diluting the sterilized cationic lipid solution of step (a) with a diluent to achieve a concentration of $\leq$ about 1 mg DMRIE/mL, and with glycerol and Vitamin E; (c) filter sterilizing a plasmid DNA solution; (d) adding the sterilized plasmid DNA solution of step (c), having a concentration of $\leq$ about 5 mg plasmid DNA/mL, to the diluted sterilized cationic lipid solution of step (b) at an ionic strength that is lower than isotonicity to form DNA/lipid complexes at a mass ratio of about 5:1 DNA to DMRIE; and (e) adjusting the DNA/lipid complexes of step (d) to about 0.9% sodium chloride, wherein the final concentration of glycerol is about 1% and of Vitamin E is about 0.01%.

According to other embodiments, the invention provides single-vial formulations of plasmid DNA/cationic lipid complexes for human clinical use prepared by any of the above processes.

In another embodiment, the invention provides a single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use comprising a cationic lipid component and a plasmid DNA component, wherein the plasmid DNA component and the cationic lipid component are combined at an ionic strength that is lower than isotonicity to form the plasmid DNA/cationic lipid complexes, and further comprising a nearly

isotonic aqueous medium, optionally containing no buffering agent except the plasmid DNA itself. The single-vial formulation may be stable in frozen, refrigerated, room temperature, or body temperature form. The single-vial formulation may be stable in frozen, refrigerated, or room temperature form for at least about 8 weeks. The single-vial formulation may further comprise about 1% glycerol and about 0.01% Vitamin E in nearly physiological saline.

In still another embodiment, the invention provides a single-vial formulation for human clinical use comprising plasmid DNA/cationic lipid complexes having storage stability in frozen, refrigerated, or room temperature form for at least about 8 weeks.

In yet another embodiment, the invention provides a single-vial formulation for human clinical use comprising plasmid DNA/cationic lipid complexes retaining *in vitro* transfection efficacy of freshly prepared plasmid DNA/cationic lipid complexes for at least about 8 weeks.

DETAILED DESCRIPTION OF THE INVENTION

The objects of the invention are achieved by controlled combination of a plasmid DNA solution with a cationic lipid solution, preferably in dilute form, at an ionic strength that is lower than isotonicity, to obtain plasmid DNA/cationic lipid complexes, which, when admixed with solutes to generate isotonicity, provide single-vial formulations suitable for human clinical use.

We have discovered that the order in which individual plasmid DNA and cationic lipid components are mixed to produce plasmid DNA/cationic lipid complexes is important to the final results, as is the ionic strength at the time of initial complex formation. Optimal *in vitro* transfection efficiency and prevention of lipid aggregation have been obtained by slow addition of a plasmid DNA solution to a cationic lipid solution at an ionic strength that is lower than isotonicity. In this scheme, we have optimized *in vitro* transfection efficiency and prevention of lipid aggregation even further by using the cationic lipid component in a dilute form upon its combination with the plasmid DNA component to form the DNA/lipid complexes.

We have also found that sterilization of lipid mixtures by filtration compromises their integrity. In contrast, we have determined that our lipid mixtures can survive sterilization by autoclave treatment. Maximum *in vitro* transfection efficiency and prevention of lipid degradation have been produced by autoclave sterilization. In this scenario, *in vitro* transfection efficiency is further maximized and lipid degradation further prevented by maintaining the lipid mixtures at higher concentrations during autoclave sterilization.

Preferred single-vial formulations of the invention have accordingly been obtained by preparing cationic lipid mixtures containing a cationic lipid constituent and a neutral lipid constituent, having a molar ratio in the range of from about 90:10 to about 10:90, preferably about 50:50. A cationic lipid solution is prepared from the cationic lipid mixture by hydrating a dried lipid film with a suitable diluent, preferably using water, to obtain a solution in highly concentrated form. The cationic lipid solution is considered to be highly concentrated by having a concentration in the range of from about 0.5 to about 5.0 M. This highly concentrated cationic lipid solution is subsequently subjected to sterilization by standard autoclave treatment, e.g., 30 minutes at 121°C, in the usual way.

Alternatively, a cationic lipid solution can be prepared and sterilized by filtration or irradiation or other appropriate means at any desired concentration, the higher concentration of cationic lipid solution being advantageous for protecting the material during sterilization by autoclave treatment.

Upon obtaining a highly concentrated cationic lipid solution, one preferably dilutes the solution, preferably using sterilized water (e.g., sterile WFI). Other suitable diluents, such as salines, can be substituted for water, and are selected on the basis of having low ionic strength, that is, lower than isotonicity. The cationic lipid solution is diluted to achieve a concentration in the range of from about 0.01 to about 1.0 M. Optionally, at this point, suitable formulatory agents may be introduced, as discussed below, particularly emulsifiers to facilitate the suspension of DNA and lipid during the combination step (infra).

Plasmid DNA, which is pharmaceutical-grade in quality, is meanwhile obtained. The plasmid DNA, in aqueous solution, such as water (e.g., sterile WFI) or other appropriate diluent, such as saline (e.g., physiological saline), is sterilized. Sterilization is preferably by filtration, for instance, through a 0.2 μm membrane filter. It is preferred that the diluent have an ionic strength that is no higher than isotonicity.

The sterilized plasmid DNA solution and diluted sterilized cationic lipid solution are usually brought to room temperature prior to combination. The DNA solution may be used at concentrations within a range that extends from about 0.05 to about 10 mg/mL. The solutions are then combined, preferably, by controlled addition of the plasmid DNA solution to the cationic lipid solution at low ionic strength with mixing to form DNA/lipid complexes. By low ionic strength is meant lower than isotonicity. Continuous mixing of the lipid solution and plasmid DNA solution as they are combined is preferred, with a further short period of mixing after the combination is complete. The mixing may be achieved by vortexing, manual agitation (shaking), mechanical mixing or stirring, or other suitable means.

DNA and lipid are combined to produce complexes at a mass ratio that is optimal for transfection efficiency, as evaluated, for example, by *in vitro* transfection assays.

The complexes are subsequently adjusted with a tonicifier to isotonicity for physiological administration. For example, adequate sterile sodium chloride stock may be added to give a final concentration of about 0.9% NaCl (i.e., physiological saline). The final formulation can next be aseptically filled into sterile depyrogenated vials, and can then be stored frozen at about -10°C to -70°C, preferably about -20°C.

At the clinic site, the DNA/lipid vials are conveniently thawed and typically mixed (e.g., by vortexing or manual agitation, i.e., shaking). They can be maintained at room temperature, and are administered advantageously within 24 hours of thawing. Each vial may deliver DNA in unit dosage form, or, alternatively, may constitute a multidose container.

For use by the physician, the single-vial formulations are provided in concentrations up to about 10 mg DNA/mL, preferably up to about 0.5 mg DNA/mL. As will be recognized by those in the field, an effective amount of DNA will vary with many factors, including the condition being treated, the characteristics of the

patient, and other factors. Typical doses will contain from about 5 mg to about 10 μg DNA, although wide variations from this range are possible while yet achieving useful results.

Other preferred single-vial formulations of the invention include physiologically acceptable excipients as called for by accepted pharmaceutical practice. These may constitute buffers, antioxidants, amino acids, emulsifiers, starches, sugars, solubilizers, surfactants, suspending agents, tonicifiers, wetting agents, etc. It has been found that the single-vial formulations provided herein maintain physiological pH without the use of buffering agents except the plasmid DNA itself. While additional buffering agents may be omitted, they may alternatively be added as occasion provides.

Preferred formulations are suitable for administration that is parenteral, that is, by any means other than oral. Parenteral administration includes injections, such as intravenous, intraarterial, intramuscular, subcutaneous, intradermal, intraperitoneal, intratumor, and interstitial injections, infusions, and by inhalation. Injections include administration through needle/syringe and catheters.

In particularly preferred embodiments, the invention provides single-vial formulations having final concentrations of 0.9% sodium chloride (i.e., physiological saline) for isotonicity, 1% glycerol as an emulsifier (and a cryoprotectant), and 0.01% Vitamin E as a preservative (and an antioxidant).

CATIONIC LIPIDS

Cationic lipid reagents that are in use today for DNA transfection are formulated as lipid vesicles or liposomes containing cationic or positively charged lipids in combination with other lipids. The formulations may be prepared from a mixture of positively charged lipids, negatively charged lipids, neutral lipids, and cholesterol or a similar sterol.

The positively charged lipid can be one of the cationic lipids, such as DMRIE, described in U.S. Patent No. 5,264,618, or one of the cationic lipids DOTMA, DOTAP, or analogues thereof, or a combination of these. DMRIE is 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide, and is preferred. See Felgner et al., J. Biol. Chem. 269:2550 (1994). DMRIE can be synthesized according to Example 1.

Neutral and negatively charged lipids can be any of the natural or synthetic phospholipids or mono-, di-, or triacylglycerols. The natural phospholipids may be derived from animal and plant sources, such as phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylserine, or phosphatidylinositol. Synthetic phospholipids may be those having identical fatty acid groups, including, but not limited to, dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine and the corresponding synthetic phosphatidylethanolamines and phosphatidylglycerols. The neutral lipid can be phosphatidylcholine, cardiolipin, phosphatidylethanolamine, mono-, di- or triacylglycerols, or analogues thereof, such as dioleoylphosphatidylethanolamine (DOPE), which is preferred. DOPE can be purchased from Avanti Polar Lipids (Alabaster, Ala). The negatively charged lipid can be phosphatidylglycerol, phosphatidic acid or a similar phospholipid analog. Other additives such as cholesterol, glycolipids, fatty acids, sphingolipids, prostaglandins, gangliosides, neobea, niosomes, or any other

natural or synthetic amphophiles can also be used in liposome formulations, as is conventionally known for the preparation of liposomes.

In a composition for preparing cationic liposomes, the cationic lipid can be present at a concentration of between about 0.1 mole % and 100 mole %, preferably 5 to 100 mole %, and most preferably between 20 and 100 mole %. The neutral lipid can be present in a concentration of between about 0 and 99.9 mole %, preferably 0 to 95 mole %, and most preferably 0 to 80 mole %. In order to produce lipid vesicles or liposomes having a net positive charge, the quantity of the positively charged component must exceed that of the negatively charged component. The negatively charged lipid can be present at between about 0 to 49 mole % and preferably 0 to 40 mole %. Cholesterol or a similar sterol can be present at 0 to 80 mole %, and preferably 0 to 50 mole %.

Lipid compositions having at least one amphipathic lipid can spontaneously assemble to form liposomes. Lipid reagents having a cationic lipid species can be prepared as cationic liposomes. The component lipids can be dissolved in a solvent such as chloroform. The mixture can be evaporated to dryness as a film on the inner surface of a glass vial. On suspension in an aqueous solvent, the amphipathic lipid molecules will assemble themselves into liposomes. See Example 2.

The liposomes can be analyzed for potency by *in vitro* transfection assays. In these assays, plasmid DNA and cationic lipid complexes are formed by mixing of the two separately diluted components. The mixture is then added to cells in culture and transfection assessed according to the procedure of Example 3.

PLASMID DNAs

The plasmid DNA required for lipid-mediated gene transfer has been widely and routinely prepared in the laboratory for many years. Sambrook, Fritsch, and Maniatis, Molecular Cloning: a Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York, 1989.

These plasmids accordingly can be selected from among prokaryotic and eukaryotic vectors, pBR322- and pUC-based vectors, and their derivatives, etc. They can utilize any of various origins of replication, for instance, prokaryotic origins of replication, such as pMB1 and ColE1, and eukaryotic origins of replication, such as those facilitating replication in yeast, fungi, insect, and mammalian cells (e.g., SV40 ori). They can incorporate any of numerous genetic elements to facilitate cloning and expression, such as selectable genes, polylinkers, promoters, enhancers, leader peptide sequences, introns, translation facilitators, Kozak sequences, polyadenylation signals, transcription terminators, 5' UTRs, 3' UTRs, etc. The selection of vectors, origins, and genetic elements will vary based on requirements and is well within the skill of workers in this art.

Genes encoding any of diverse structural proteins (or peptides, polypeptides, glycoproteins, phosphoproteins, amidated proteins, etc.) can be inserted into the plasmids for delivery into cells. These genes may constitute genomic DNA, cDNA, synthetic DNA, polynucleotide, oligonucleotide, etc. sequences. Transfer of mRNA, antisense oligomers, and triple helix agents are also expressly contemplated as falling within the scope of the present invention. These sequences may be obtained using chemical synthesis or

gene manipulation techniques. They can be inserted into plasmids, and the plasmids subsequently introduced into host cells for propagation.

Host cells can be selected from among prokaryotes and eukaryotes, including bacterial, yeast, fungi, insect and mammalian cells. Preferred hosts are bacteria, such as E. coli. Any suitable strain of E. coli is contemplated.

Propagation of plasmid DNA-containing hosts can be carried out using known processes. Such processes may utilize incubators, bioreactors, fermentors, etc., according to batch fermentation, fed batch fermentation, continuous culture, Type I, II, and III fermentation, aseptic fermentation, consortium fermentation, protected fermentation, etc. Fitting the conditions (e.g., medium, temperature, pH, hours, agitation, aeration, etc.) for propagation to the circumstances is empirical and well within the skill of those in the art.

Purification of plasmid DNA to pharmaceutical grade quality may proceed using well established processes. Alternatively, processes for production of pharmaceutical grade plasmid DNA disclosed in Example 8 are preferred. Additionally, processes for reducing RNA concentrations in cell lysates using diatomaceous earth materials also according to Example 8 are also preferred in purifying plasmid DNA to pharmaceutical grade standards.

REGULATORY PROCESSES

Gene therapy requires approvals from several different regulatory agencies in the United States, including the Food and Drug Administration (FDA). The FDA oversees and regulates inter alia that medical drugs are safe and efficacious and how they are manufactured (e.g., GMP). Similar approvals are required by most foreign countries. The single-vial formulations of the invention comply with these regulatory processes.

APPLICATION

In one application, DMRIE/DOPE cationic lipid mixtures are prepared at from about 90:10 to about 10:90 molar ratio, preferably at about 50:50 molar ratio. A cationic lipid solution is prepared from the cationic lipid mixture by hydrating a dried lipid film with a suitable diluent, preferably with water, at $\geq$ about 2 mg DMRIE/mL, preferably at about 2 to about 10 mg DMRIE/mL, most preferably at about 8 mg DMRIE/mL. This highly concentrated cationic lipid solution is subsequently subjected to sterilization by standard autoclave treatment, e.g., 30 minutes at 121 °C, in the usual way.

Upon obtaining an autoclaved highly concentrated DMRIE/DOPE lipid solution, one then dilutes the solution, preferably with sterilized water (e.g., sterile WFI), to $\leq$ about 2 mg DMRIE/mL, preferably to $\leq$ about 1 mg DMRIE/mL, most preferably to $\leq$ 0.2 mg DMRIE/mL. Other suitable diluents, such as salines, can be substituted for water, and are selected on the basis of having low ionic strength, that is, lower than isotonicity. Optionally, at this point, suitable formulatory agents may be introduced, advantageously by addition into the diluent, for subsequent admixture into the cationic lipid solution. For example, emulsifiers may be introduced, preferably glycerol, in amounts to provide a final concentration in the single-vial

formulation of from about 0.5 to about 5% glycerol, preferably 1% glycerol. Additionally, preservatives may also be added at this time, preferably Vitamin E, in amounts to give a final concentration in the single-vial formulation of from about 0.005 to about 0.05% Vitamin E, preferably about 0.01% Vitamin E.

Plasmid DNA that has been purified to pharmaceutical-grade quality is meanwhile obtained. A process for the production of pharmaceutical-grade plasmid DNA is provided in Example 4. The plasmid DNA, in aqueous solution, such as water (e.g., sterile WFI) or other appropriate diluent, such as saline (e.g., physiological saline), is sterilized. Sterilization is preferably by filtration, for instance, through a 0.2 μm membrane filter. It is preferred that the diluent have an ionic strength that is no higher than isotonicity.

The sterilized plasmid DNA solution and diluted sterilized DMRIE/DOPE lipid solution are usually brought to room temperature prior to combination. The DNA may be used at concentrations within a range that extends from concentrations that are quite high, for example, 10 mg/mL, to concentrations that are quite low, for example, 0.02 mg/ml. The solutions are then combined, preferably, by controlled addition of the plasmid DNA solution to the DMRIE/DOPE solution with mixing to form DNA/lipid complexes at low ionic strength. Low ionic strength means lower than isotonicity.

DNA and lipid are combined to produce complexes at a mass ratio of from about 50:1 to about 1:10 DNA to DMRIE, preferably at a mass ratio of from about 10:1 to about 1:5 DNA to DMRIE, most preferably at a mass ratio of about 5:1 DNA to DMRIE.

Subsequently, the complexes are adjusted with solutes to isotonicity for physiological administration. In particularly preferred embodiments, the invention provides single-vial formulations having final concentrations of 0.9% sodium chloride, 1% glycerol, and 0.01% Vitamin E.

UNIVERSALITY

Single-vial formulations of the invention have been successfully tested with several different plasmid DNAs, for example, plasmids containing reporter genes, for instance, luciferase and ß-galactosidase, and plasmids operatively encoding polypeptides suitable for human gene therapy, e.g., HLA-B7 and human IL-2. See Examples 5-10. Additionally, we have applied the present invention to numerous diverse cationic lipid species and cationic lipid mixtures, for example, DMRIE, DMRIE/DOPE, DOSPA, DOSPA/DOPE, HP-DORIE, HP-DORIE/DOPE, τ-MU-DMRIE, τ-MU-DMRIE/DOPE, τ-MC-DMRIE, τ-MC-DMRIE/DOPE, δ-Ser-DMRIE, δ-Ser-DMRIE/DOPE, βAE-DMRIE, βAE-DMRIE/DOPE, Arabinose-TU-τ-DMRIE, Arabinose-TU-τ-DMRIE/DOPE, Galactose-TU-τ-DMRIE, Galactose-TU-τ-DMRIE/DOPE, Glucose-TU-τ-DMRIE, and Glucose-TU-τ-DMRIE/DOPE. These results demonstrate the universality of the present invention.

SAFETY

Single-vial formulations of the invention containing DNA/DMRIE-DOPE at a mass ratio of 5:1 DNA to cationic lipid in 0.9% sodium chloride with 1% glycerol and 0.01% Vitamin E demonstrated no signs of toxicity in acute intravenous toxicity studies in mice, repeat dose safety studies in mice, and repeat dose safety studies in cynomolgus monkeys.

STABILITY

The above single-vial formulations were found to be stable. Accelerated testing evidenced full stability over a time course of 8 weeks at -20°C, refrigerator, and room temperature. This testing showed full stability over a time course of one month at body temperature. These studies demonstrated the utility of the antioxidant component of the formulation. Example 11.

HUMAN GENE THERAPY

The single-vial formulations of the invention are suitable for human clinical use *in vivo* (Example 12-13) or *ex vivo* (see U.S. Patent No. 5,399,346 to Anderson et al. for "Gene Therapy").

Particular aspects of the invention may be more readily understood by reference to the following examples, which are intended to exemplify the invention, without limiting its scope to the particular exemplified embodiments.

EXAMPLE 1

PREPARATION OF 1,2-DIMYRISTYLOXYPROPYL-3-DIMETHYL-HYDROXYETHYL AMMONIUM BROMIDE (DMRIE)

DMRIE was synthesized using minor modifications of the procedure developed for the synthesis of DOTMA (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413 (1987)). Thus, 3-dimethylamino-1,2-propanediol was condensed with myristyl mesylate employing basic catalysis to generate the corresponding diether. Subsequent to chromatographic purification of this lipophilic amine, quatranization was effected by treatment with 2-bromoethanol at elevated temperatures. The chromatographically purified product exhibited IR, TLC, and elemental analyses consistent with those predicted for the desired hydroxyalkyl ammonium salt.

EXAMPLE 2

CATIONIC LIPOSOME PREPARATION

DMRIE was synthesized according to Example 1. DOPE was purchased from Avanti Polar Lipids (Alabaster, Ala). Cationic liposomes were prepared by mixing a chloroform solution of the lipids in a sterile glass flask. The solvent was removed by evaporation under reduced pressure to produce a dried lipid film. Vials were placed under vacuum overnight to remove any solvent traces. The lipid mixture was hydrated by addition of sterile water for injection.

EXAMPLE 3

IN VITRO TRANSFECTION PROTOCOLS

Plasmid DNA/cationic lipid complexes were prepared by mixing an aliquot of an polynucleotide solution with an aliquot of a liposome solution at room temperature. Different ratios of positively charged liposomes to polynucleotides can be used to suit the need. The methods are a modification of those described in Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413 (1987), and Felgner and Holm, Focus 11(2) Spring, 1989.

Transfections were carried out in 96-well plates, as follows:

(1) The wells of a 96-well microtiter plate were seeded with 20,000 to 40,000 cells per well;

(2) Single-vial formulations of plasmid DNA/cationic lipid complexes were prepared in sterile vials or tubes;

(3) Dilutions of the single-vial formulations prepared as in (2) were carried out by serial administration into cell culture medium (with or without fetal calf serum) distributed in appropriate volumes in 96-well plates;

(4) The cell culture medium covering the cells that had been seeded as in (1) and grown to confluence was removed by aspiration;

(5) The cells were washed with an additional volume of cell culture medium without fetal calf serum, and the wash medium was removed by aspiration;

(6) A volume of the diluted plasmid DNA/cationic lipid complexes was added to the washed cells in a well of the microtiter plate; the volume transferred usually consisted of 80 to 100 μL;

(7) Adequate 50% fetal calf serum was added to each well to bring the concentration of serum in the wells to 10%;

(8) The plates were incubated at 37°C (5% CO_2). At 12 and 24 hours post transfection, an aliquot of 10% serum in Opti-MEM® reduced-serum media (GIBCO BRL Life Technologies, Gaithersburg, MD) was added to each well;

(9) At the end of the incubation (usually 48 or 72 hours), the medium covering the cells or a whole cell lysate was assayed for expression activity.

Where β-galactosidase was the reporter gene, the expression was monitored colorimetrically, using chlorophenyl red-β-galactopyranoside (CPRG) as a substrate, reading the plates with a microtiter reader at 405 nm.

EXAMPLE 4

PROCESS FOR THE PURIFICATION OF PHARMACEUTICAL-GRADE PLASMID DNA

Cell Lysis. A cell paste was resuspended completely in 6 mL per gram wet bacterial weight of cold Solution I (61 mM glucose + 25 mM Tris buffer pH 8.0 + 10 mM EDTA at 5°C) with stirring at room temperature. To this solution 12 mL per gram wet bacterial weight Solution II (0.2 N NaOH + 1% SDS) was added and mixed end-over-end until homogeneous. This was incubated on wet ice for approximately 10 minutes. To the lysed cell solution, 9 mL per gram wet bacterial weight of cold Solution III (3.0 M potassium acetate pH 5.0 at 5°C) was added, mixed end-over-end until a white flocculent precipitate appeared, and incubated on wet ice for approximately 5 minutes.

Filtration. The cell debris was removed from the lysate by filtration, centrifugation or sedimentation. The supernatant was collected and clarified by adding approximately 25 g/l Celite® diatomaceous earth and filtering through a (preferably precoated) filter membrane (Whatman # 1, 113 or equivalent) arranged in a table top Buchner funnel. Alternatively, the cell debris was removed from the lysate by direct Celite® aided filtration. In this case, approximately 90 g/l Celite® diatomaceous earth was added directly to the lysis

solution and mixed by swirling until homogenous. The lysate was then filtered through a (preferably precoated) filter membrane (Whatman # 1, 113 or equivalent) arranged in a table top Buchner funnel.

DNA Precipitation. Polyethylene glycol (PEG, e.g., PEG-8000) was added to the filtrate to 5-15% (w/v), plus NaCl to 0.3-1.5 M. The PEG suspension was stirred preferably overnight at 2-8°C. The DNA precipitate was collected by adding approximately 25 g/l of Celite® diatomaceous earth to the PEG suspension and filtering through a (preferably precoated) filter membrane arranged in a table top Buchner funnel. The DNA precipitate was captured in the Celite® cake and recovered by suspending the cake in TE buffer (0.01 M Tris-base pH 8.0 + 0.001 M EDTA).

RNA, Protein and Lipopolysaccharide Removal. Ammonium acetate was added to the TE buffer to 2.5 M and stirred for approximately 30 minutes at 2-8°C. The suspension, which still contained diatomaceous earth, was filtered through a (preferably precoated) filter membrane arranged in a table top Buchner funnel. The DNA filtrate was then optionally clarified by sub-micron filtration.

Final DNA Precipitation. A final DNA precipitation was performed with 0.6 volumes of cold isopropanol for a minimum of 2 hours at -20°C. The precipitated DNA was centrifuged in a Sorvall table top centrifuge for 30 minutes at 2000 x g or equivalent. The DNA pellets were resuspended in column buffer prior to gel filtration chromatography.

Gel Filtration Chromatography. A Pharmacia S-1000 tandem size exclusion column, DNA exclusion limit of 20,000 bp, (Pharmacia, Piscataway, NJ) was poured. The S-1000 matrix was an inert and highly stable matrix that was prepared by covalently cross-linking allyl dextran with N,N'-methylenebisacrylamide. The column was poured in two Pharmacia XK26/100 columns (Pharmacia, Piscataway, NJ) with a final bed height of 80-85 cm (2.6x80cm) resulting in a total column volume of approximately 900 mL and a total length of approximately 160 cm. The columns were individually pressure packed in one direction, reversed and connected in series for equilibration and operation. The column was equilibrated in column buffer and run at an appropriate flow rate. Cleared lysate plasmid DNA was filtered through a 0.2 μm filter and loaded onto the column. Column operation and fractionation were automated with a Pharmacia FPLC (Pharmacia, Piscataway, NJ). Fractions (approximately 0.5 - 5% of column volume) were collected over the product elution zone and analyzed by 0.8% agarose gel electrophoresis. Appropriate fractions were pooled and precipitated with 2 volumes of cold ethanol. This column purified DNA was stored frozen at -20°C until needed for preparation of bulk plasmid DNA.

Bulk Plasmid DNA Preparation. The ethanol precipitated, column purified DNA was spun at maximum speed in a Sorvall table top centrifuge for 30 minutes at 4-10°C or equivalent. The pellets were air-dried and pooled. The pooled pellets were resuspended in vehicle, for example, sterile WFI or physiological saline. The DNA was then filtered through a 0.2 μm filter into a pyrogen-free container. Samples were optionally taken for quality control testing and the remainder stored frozen at -10°C to -70°C, preferably at -20°C.

EXAMPLE 5

HLA-B7/DMRIE-DOPE COMPLEXES

HLA-B7 plasmid DNA/DMRIE-DOPE lipid complexes, intended for use in human gene therapy, demonstrated *in vitro* potency as single-vial formulations of the invention. See Nabel et al., Human Gene Therapy 3:399 (1992); Nabel et al., Proc. Natl. Acad. Sci. USA 90:11307 (1993); PCT Patent Application WO# 94/29469; Nabel et al., Human Gene Therapy 5:57 (1994); Vogelzang et al., Human Gene Therapy 5:1357 (1994); Hersh et al., Human Gene Therapy 5:1371 (1994); and Rubin et al., Human Gene Therapy 5:1385 (1994).

An HLA-B7 encoding plasmid was constructed of about 5000 bp in size. It derived from a pBR322-based plasmid containing a bacterial origin of replication. It encoded the heavy (human HLA-B7 cDNA) and light (chimpanzee β-2 microglobulin cDNA) chains of a Class 1 MHC antigen designated HLA-B7. These two proteins were expressed on a bi-cistronic mRNA. Eukaryotic cell expression of this mRNA was dependent on a Rous Sarcoma Virus (RSV) promoter sequence derived from the 3' Long Terminal Repeat (LTR). Expression was also dependent on a transcription termination/polyadenylation signal sequence derived from the bovine growth hormone gene. Expression of the heavy chain was regulated by the 5' cap-dependent protein translation start site. Expression of the light chain was regulated by a Cap Independent Translational Enhancer (CITE) sequence derived from the Encephalomyocarditis Virus. The plasmid also encoded a kanamycin resistance gene derived from Tn903.

EXAMPLE 6

PHARMACEUTICAL-GRADE PURIFIED HLA-B7 PLASMID DNA

The HLA-B7 encoding plasmid was purified to pharmaceutical-grade standards as determined by the criteria given in Table 1 below.

TABLE 1: HLA-B7 PLASMID DNA QUALITY CONTROL CRITERIA

TEST	SPECIFICATION	METHOD
Size Identity	Approximates: 4900 bp	Agarose Gel Electrophoresis
Restriction Sites	Approximates predicted: XhoI/XbaI - 3500 & 1400 bp, BglII/XhoI - 2100, 1700 & 1000 bp	Agarose Gel Electrophoresis
Circular Plasmid DNA	> 95% of visualized nucleic acid	Agarose Gel Electrophoresis
A260/A280 Ratio	1.75 to 2.00	UV Absorbance
E coli DNA	< 0.01 μg/μg plasmid DNA	Southern Slot Blot
RNA	Non-visualized on gel	Agarose Gel Electrophoresis
Protein	Undetectable	BCA Colorimetric Assay
Pyrogenicity	Not pyrogenic at 5 μg/Kg rabbit body weight	Rabbit Pyrogen Assay
Endotoxin	< 0.1 EU/μg plasmid DNA	Limulus Amebocyte Lysate (LAL) Assay
Sterility	No growth through 14 days	Fluid Thioglycollate Assay
Potency	50-200% of reference	*In Vitro* Transfection/ Fluorescence
General Safety Test	Passes	per 21 C.F.R. 610.11

EXAMPLE 7

IN VITRO POTENCY OF HLA-B7/DMRIE-DOPE COMPLEXES

Transfection efficiency was determined by HLA-B7 gene expression in SW480 cells, a human colon adenocarcinoma cell line, ATCC # 228-CCL, or UM449 cells, a human melanoma cell line, Alfred Chang, University of Michigan, following *in vitro* transfection using HLA-B7 plasmid DNA/DMRIE-DOPE lipid complexes formed by the methodology of the invention in 0.9% sodium chloride with 1% glycerol and 0.01% Vitamin E.

From 200,00 to 400,000 UM449 cells were seeded per well into a 6-well plate the day before transfection. Cells were a >75% confluent monolayer prior to transfection. The cells were transfected with 10 μg plasmid DNA in the presence of 2 μg DMRIE (synthesized in house) and 2 μg DOPE (purchased from Avanti Polar Lipids, Alabaster, Ala). The cells were incubated at 37°C, 5% CO_2 throughout. Reduced serum media, e.g., Opti-MEM® reduced-serum media (GIBCO BRL Life Technologies, Gaithersburg, MD), supplemented with fetal calf serum, was added to the cells 1-4 hours and 24 hours post-transfection. Cells were harvested 48 hours post-transfection.

HLA-B7 expression on the cell surface was measured by labelling with anti-HLA-B7 mouse antibody, followed by a fluorescent secondary antibody (anti-mouse IgG monoclonal antibody R-phycoerythrin conjugate). Immunofluorescent staining of the cells was analyzed by flow cytometry. A two-fold increase in mean fluorescence intensity was observed for transfected cells in contrast to negative controls (non-transfected cells or cells transfected with an irrelavant gene). Potency was equivalent to that of freshly prepared plasmid DNA/cationic lipid complexes.

EXAMPLE 8

IL-2/DMRIE-DOPE COMPLEXES

IL-2 plasmid DNA/DMRIE-DOPE lipid complexes, intended for use in human gene therapy, demonstrated *in vitro* potency as single-vial formulations of the invention.

A plasmid encoding IL-2 was constructed of about 5000 bp in size. It derived from a pUC-based plasmid containing a bacterial origin of replication. It encoded an IL-2 fusion protein. The protein was constructed by cloning a portion encoding a short segment of the 5' untranslated region and the first six amino acids of the leader peptide of the rat insulin II gene 5' of the human IL-2 coding sequence minus the first two amino acids of its leader peptide. This fusion protein was placed under the eukaryotic transcriptional control of the cytomegalovirus (CMV) immediate early 1 promoter/enhancer sequence. This sequence facilitated expression of a composite mRNA containing a 5' untranslated sequence from the CMV immediate early 1 gene, including the 800+ bp intron, the IL-2 fusion protein coding sequence, and a 3' untranslated sequence derived from the bovine growth hormone gene having transcription termination/polyadenylation signal sequence. The plasmid also encoded a kanamycin resistance gene derived from Tn903.

EXAMPLE 9

PHARMACEUTICAL-GRADE PURIFIED IL-2 PLASMID DNA

The IL-2 encoding plasmid was purified to pharmaceutical-grade standards as determined by the criteria given in Table 2 below.

TABLE 2: IL-2 PLASMID DNA QUALITY CONTROL CRITERIA

TEST	SPECIFICATION	METHOD
Size Identity	Approximates 4900 bp	Agarose Gel Electrophoresis
Restriction Sites	Approximates predicted: EcoRI - 3000 & 1900 bp, NcoI - 3700 & 1200 bp	Agarose Gel Electrophoresis
Circular Plasmid DNA	> 95% of visualized nucleic acid	Agarose Gel Electrophoresis
A260/A280 Ratio	1.75 to 2.00	UV Absorbance
E coli DNA	< 0.01 μg/μg plasmid DNA	Southern Slot Blot
RNA	Non-visualized on gel	Agarose Gel Electrophoresis
Protein	< 0.016 μg/μg plasmid DNA	Protein Slot Blot
Residual Ethanol	< 500 ppm	Gas Chromatography
Pyrogenicity	Not pyrogenic at 5 μg/Kg rabbit body weight	Rabbit Pyrogen Assay
Endotoxin	< 0.1 EU/μg plasmid DNA	Limulus Amebocyte Lysate (LAL) Assay
Sterility	No growth through 14 days	USP Direct Transfer
Potency	50-200% of reference	*In Vitro* Tranfection/ ELISA
General Safety Test	Passes	USP General Safety Test

EXAMPLE 10

IN VITRO POTENCY OF IL-2/DMRIE-DOPE COMPLEXES

Transfection efficiency was determined by IL-2 gene expression in B16F0 cells, a mouse melanoma cell line, ATCC # CRL 6322, following *in vitro* transfection using IL-2 plasmid DNA/DMRIE-DOPE lipid complexes formed by the methodology of the invention in 0.9% sodium chloride with 1% glycerol and 0.01% Vitamin E.

From 200,00 to 400,000 B16F0 cells were seeded per well into a 6-well plate the day before transfection. Cells were a >75% confluent monolayer prior to transfection. The cells were transfected with 2.5 μg plasmid DNA in the presence of 0.5 μg DMRIE (synthesized in house) and 0.5 μg DOPE

(purchased from Avanti Polar Lipids, Alabaster, Ala). The cells were incubated at 37°C, 5% CO_2 throughout. A reduced serum medium, e.g., Opti-MEM® reduced-serum media (GIBCO BRL Life Technologies, Gaithersburg, MD), supplemented with fetal calf serum, was added to the cells at commencement of transfection and 24 hours post-transfection. Cell supernatant was harvested 48 to 80 hours post-transfection.

IL-2 expression in the cell supernatant was measured by an enzyme amplified sensitivity immunoassay (Medgenix ELISA, Medgenix Diagnostics, Fleurus, Belgium). Potency was equivalent to that of freshly prepared plasmid DNA/cationic lipid complexes.

EXAMPLE 11

STABILITY OF IL-2/DMRIE-DOPE COMPLEXES

The stability of IL-2 plasmid DNA/DMRIE-DOPE lipid complexes formed by the methodology of the invention and stored in 0.9% sodium chloride with 1% glycerol and 0.01% Vitamin E was evaluated at -20°C, 2°C, 25°C, and 37°C. An additional preparation of DNA/DMRIE-DOPE complexes in 0.9% sodium chloride with 1% glycerol and no Vitamin E was evaluated at the 37°C storage temperature. The stability of the materials in the study was analyzed by a number of methods, including 96-well transfection assay. The results of the study showed that IL-2/DMRIE-DOPE complexes and free DNA in 0.9% sodium chloride with 1% glycerol and 0.01% Vitamin E retained full stability over 57 days of storage at -20°C and 2°C. At 25°C IL-2/DMRIE-DOPE complexes retained apparent full activity over 57 days of storage; free DNA showed a half-life of 500 days for conversion from circular to linear form at this temperature. At 37°C, IL-2/DMRIE-DOPE complexes showed a half-life of 34 days; free DNA was converted from circular to linear form with a 290 day half-life at this temperature. In comparison, in the absence of Vitamin E, IL-2/DMRIE-DOPE complexes showed a half-life of 12 days at 37°C; and free DNA was converted from circular to linear form with a 108 day half-life at this temperature. Therefore, the presence of Vitamin E at the level of 0.01% in the subject embodiment provided 2.7 to 3 times greater stability to the components of the vial.

EXAMPLE 12

PREPARATION OF IL-2/DMRIE-DOPE COMPLEXES

A. LIPID FORMULATION and AUTOCLAVE STERILIZATION.

1. Preparation of the DMRIE/DOPE Bulk Solution.

Using an analytical balance, the batch amount of DMRIE Br was weighed out.

Working in a ventilated laminar flow hood, the DMRIE Br was placed in a clean 50-mL round bottom flask.

Using a 5-mL glass pipet, five (5) mL of chloroform was added to the round bottom flask from above. The flask was swirled to dissolve the DMRIE Br.

Using a SMI pipet, the batch amount of DOPE was added.

The neck of the round bottom flask was rinsed with about five (5) mL of chloroform and the contents swirled gently for at least one minute to mix. Any solution adhering to the neck after swirling was rinsed into the flask with additional chloroform.

Using a rotary evaporator, the chloroform was removed from the solution from above. The flask was kept on the rotary evaporator until condensate was no longer visible in the condenser.

A desiccator was thoroughly wiped down with alcohol and placed in a ventilated laminar flow hood. The round bottom flask from above was placed in the desiccator. The desiccator was connected to a vacuum pump, the desiccator evacuated, and the round bottom flask maintained under vacuum for at least 12 hours.

After at least 12 hours exposure to vacuum, the desiccator was isolated from the vacuum pump.

Working in the ventilated laminar flow hood, the desiccator was connected to a nitrogen gas source, the gas turned on, and the vacuum released. When the desiccator was filled with nitrogen gas, the gas source was removed.

The round bottom flask was removed from the desiccator. Using a sterile disposable pipet, sterile water for injection was added to the flask. The flask was capped, the liquid swirled, and vortexed for at least 5 minutes or until rehydration was achieved.

2. Preparation of DMRIE/DOPE bulk lipid mixture vials for autoclave treatment.

The solution from above was transferred in ~0.5 mL aliquots to clean 2-mL Type 1 glass vials. Each vial was capped with a clean teflon-coated gray butyl stopper, and the cap secured with an aluminum crimp.

The filled vials from above were autoclaved using a standard liquid cycle on the autoclave (no less than 121°C for 30 minutes). If autoclaved vials were not used immediately, they were stored at 2 to 8°C. The autoclaved vials were not held at room temperature for more than 6 hours.

B. PLASMID DNA FORMULATION.

1. Sterile filtration of IL-2 Plasmid DNA Standard Bulk Solution.

Working in a ventilated laminar flow hood, the plasmid DNA standard bulk solution was filtered through a 0.2 μm sterile filtration unit. The filtrate was collected in a sterile disposable tube. The tube was stored at 15 to 30°C.

C. PLASMID DNA/DMRIE-DOPE LIPID MIXTURE FORMULATION AND FILL.

1. Preparation of the DMRIE/DOPE lipid solution.

Working in a ventilated laminar flow hood with all materials at room temperature (15 to 30°C), sterile water for injection was transferred into a sterile disposable 250-mL bottle.

An appropriate amount of stock glycerin and an appropriate amount of stock Vitamin E was added to the bottle from above and swirled to mix.

An appropriate amount of autoclave sterilized DMRIE/DOPE bulk lipid mixture from above was added to the solution from above and swirled to mix.

An appropriate amount of the filtered IL-2 plasmid DNA solution from above was added to the DMRIE/DOPE lipid solution from above. The bottle was capped and the mixture was swirled vigorously by hand for at least one minute.

An appropriate amount of stock (5%) sodium chloride injection was added to the IL-2 plasmid DNA/DMRIE-DOPE lipid complex solution from above. The bottle was capped and the mixture was swirled vigorously by hand for at least 1 minute.

2. Fill/Finish.

Working in a ventilated laminar flow hood, 1.2 mL of the IL-2 plasmid DNA/DMRIE-DOPE lipid complex from above was aseptically filled into prepared 2-mL type I glass vials using a Drummond Pipet-Aid and sterile disposable 2-mL pipets.

The vials were sealed with prepared 13 mm teflon-coated gray butyl stoppers and 13 mm tab-top aluminum caps. All vial caps were crimped with a hand crimper.

EXAMPLE 13

IL-2/DMRIE-DOPE COMPLEXES IN PHASE I HUMAN CLINICAL TRIALS

Direct intratumor injection of plasmid DNA expression vectors provides a method for the introduction of IL-2 genes into the tumor. In the Phase I study proposed in this protocol, the sponsor tests for safety and dose optimization of the direct gene transfer approach for delivering the IL-2 gene directly into solid tumors and lymphomas. Expression of the IL-2 gene is confirmed. The IL-2 produced should elicit an immunologic antitumor response which in turn may lead to a systemic immunological elimination of other tumor cells. Dose responses are correlated with specific immune responses.

Specifically, the phase I protocol is designed:

1) to minimize the risks to the patient;
2) to derive the maximum information regarding the expression of recombinant genes *in vivo*;
3) to maximize potential benefit to the patient; and
4) to gain new knowledge regarding gene delivery to tissues in vivo and the immune response to tumors.

The objectives of the clinical plan include:

1) To confirm the *in vivo* expression of the IL-2 gene in the tumor cells.
2) To determine the safety and toxicity of direct intralesional injections of increasing amounts of a DNA/lipid mixture, IL-2/DMRIE-DOPE, into patients with advanced malignancy.
3) To determine the biological activity and pharmacokinetics of treatment with IL-2/DMRIE-DOPE.
4) To characterize the clinical response to escalating doses of the study drug by assessing the size of the injected tumor and of other tumor masses that may be present.

The product is composed of plasmid DNA coding for IL-2, formulated in an injection vehicle with the cationic lipid mixture DMRIE/DOPE. When introduced into the target tumor tissue, the lipid facilitates transfection of cells with the plasmid. On introduction of the plasmid into cells, the recombinant gene is expressed. The lipid mixture is a combination of two compounds: DOPE (CAS name: 1,2-dioleoyl-sn-glycero-

3-phosphoethanolamine) and DMRIE synthesized as DMRIE-Br (CAS name: (+/-)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradexyloxy)-1-propanaminium bromide) which are assumed to be rapidly metabolized.

The plasmid/lipid mixture and injection vehicle are produced in accordance with the methodology of the invention. The plasmid DNA is formulated with DMRIE/DOPE lipid mixture in the injection vehicle which constitutes 1% glycerol and 0.01% Vitamin E in normal aqueous saline (0.9% sodium chloride in sterile water for injection). The dosage form is delivered by injection into solid tumor tissue.

The concentration of plasmid DNA and DMRIE/DOPE in each dose package is specified in the following table:

TABLE OF DOSE PACKAGES

Dose Package	Plasmid DNA concentration (mg/mL)	Mass dried DMRIE/DOPE (mg/mL)	Volume sterile Injection vehicle (mL)
10 μg	0.01	0.004	1.2
30 μg	0.03	0.012	1.2
100 μg	0.10	0.04	1.2
300 μg	0.30	0.12	1.2

This is a Phase I open-label study in which up to 25 patients are enrolled for injections directly into tumor nodules with a lipid-formulated IL-2 plasmid complex. Solid tumors (excluding bony tumors), and metastases of malignant melanoma, renal cell carcinoma, and hepatic metastases of advanced colorectal carcinoma, and lymphomas are the tumor types to be evaluated.

Eligible patients have a primary tumor nodule injected several times at specified intervals with a specified dose of the study drug (see below). There are four groups with 5 patients each treated at the prescribed dose (10, 30, 100 or 300 μg), with a group of 5 patients retreated at the maximum tolerated dose (MTD), or at 300 μg if the MTD is not reached. The highest dose that does not yield Grade 3 or higher toxicities is considered the MTD. All toxicities are graded according to the World Health Organization (WHO) Recommendations for Grading of Acute and Sub-Acute Toxic Effects.

TABLE OF SPECIFIED DOSES

Dosage Group	No. Of Patients Per Group	Dose Per Treatment	Total No. Of Treatments Per Patient	Days Between Treatments
I	5	10 μg	6	7
II	5	30 μg	6	7
III	5	100 μg	6	7
IV	5	300 μg	6	7
V	5	MTD	6	7

The study drug is administered and toxicities are monitored. Tumor lesions are selected for treatment if they are accessible to intratumor administration by direct needle injection. These metastatic lesions are located at any accessible site such as skin, nodes, lung, liver, soft tissues etc. Bony tumors are excluded. The amount of study drug material injected into each tumor is based on the algorithm outlined below. The prescribed dose (10, 30, 100 or 300 μg) is thawed and diluted with injection vehicle to the appropriate volume. If necessary, the study drug is injected with the aid of sonographic or CAT scan visualization of the metastasis. Prior to injection, following placement of the needle, gentle aspiration is applied to the syringe to ensure that no material is injected intravenously. After injection of the drug and with the needle still in place, the dead space is flushed with 0.25-0.50 mL of sterile normal saline (0.9% sodium chloride in sterile water for injection).

Tumor Diameter (cm)	Volume of Injection (cc)
1.0-1.5	1.0
1.6-2.0	2.0
2.1-3.0	3.0
3.1-X	4.0

Vital signs are measured every 15 minutes at the start of, during, and after the injection for at least 2 hours or until the patient is stable. If the systolic blood pressure drops below 80 mm Hg, the injection is terminated immediately and the patient is closely monitored and treated appropriately until blood pressure is normalized.

Patients are closely monitored for toxicity for 3-4 hours post injection, then 24 hours and 7 days after the first and second injections. For injections 3-6, patients are monitored for 3-4 hours post injection then 7 days post injection as long as they have experienced no toxicity during the 4 and 24 hour observation periods following injections 1 and 2.

TABLE OF SCHEDULE FOR POST-INJECTION MONITORING

Treatment #	3-4 Hrs	24 Hrs	7 Days	14 Days
1	X	X	X	---
2	X	X	X	---
3	X	---	X	---
4	X	---	X	---
5	X	---	X	---
6	X	---	X	X

Before each subsequent injection, patients are evaluated for toxicities from the prior injection and injected with the next dose only if no Grade 3 or higher toxicity occurs. A tumor sizing is done at each intratumoral injection of the nodule. If the tumor shrinks to a point where it can no longer be injected, subsequent doses are administered into another tumor nodule if any are present.

After the 6th injection, patient follow-up includes evaluations with tumor sizings at weeks 8 and 16. After the week 16 visit, patients are evaluated a minimum of every 4 months.

If a patient experiences stable disease or a partial response (see below) at 4-8 weeks after the last injection of their initial course, he/she may receive an additional course of treatment identical to the first course of treatment or the next higher dose. The patient must, however, continue to meet the entry criteria.

Adverse events are monitored, and patients are removed from the study if unacceptable toxicity (Grade III or IV) develops.

Classical pharmacological studies of drug distribution, half time, metabolism, and excretion are not entirely relevant to *in vivo* gene injection and expression. However, the fate of the plasmid and detection of the gene product (IL-2) are relevant to the development of this agent. In addition, immune activation is important. Therefore, as part of the measurement of the efficacy of this study, successful gene transfer and expression is evaluated by molecular and immunological analyses. The following parameters are measured to evaluate the tumor transfection and expression of IL-2: 1) the presence of DNA from the IL-2 gene is assessed by PCR amplification of cells obtained by biopsy of the treated site after the injection of the study drug, 2) immunohistochemical staining of tumor biopsy samples is used to assess immunologic response and soluble IL-2 expression, 3) serum IL-2 levels are measured pre-treatment and 2 times post the start of therapy, however, the detection of serum IL-2 levels is not anticipated due to IL-2 instability, 4) PCR analysis of peripheral blood samples is used to test for the presence of plasmid DNA after the start of treatment and compared to pre-therapy, but detection of the gene in peripheral blood samples is not anticipated, 5) the cellular immune response is evaluated by measuring baseline and post-treatment IL-2 induced activation of PBMC by thymidine uptake assay and NK/ LAK response in peripheral blood pre-therapy and post-therapy, and 6) an attempt is made to excise tumor tissue from another site prior to treatment for diagnosis,

immunochemistry, cryo-preservation and to evaluate peripheral blood lymphocyte immunological reactions to the tumor before and after treatment.

As an additional part of the evaluation of the efficacy of this study, the clinical response is measured. Standard oncologic criteria are applied to determine whether or not a patient responds to the study drug. All tumor measurements are recorded in centimeters and constitute the longest diameter and the perpendicular diameter at the widest portion of the tumor. The tumor response definitions listed below are used to compare current total tumor size to pre-treatment total tumor size.

There is a complete tumor response upon disappearance of all clinical evidence of active tumor for a minimum of four weeks, and the patients is free of all symptoms of cancer.

There is a partial tumor response upon fifty percent (50%) or greater decrease in the sum of the products of all diameters of measurable lesions. These reductions in tumor size must endure for a minimum of four weeks. No simultaneous increase in the size of any lesion or appearance of new lesions may occur. The appropriate diagnostic tests used to demonstrate the response must be repeated four weeks after initial observation in order to document this duration.

There is stable disease upon less than 50% decrease in the sum of the products of all diameters of measurable lesions, or an increase in the tumor mass less than 25% in the absence of the development of new lesions.

There is progressive disease upon tumor progression as defined if one or more of the following criteria are met: 1) appearance of any new lesions(s), 2) increase in tumor size of $\geq 25\%$ in the sum of the products of all diameters of measurable lesions, 3) significant clinical deterioration that cannot be attributed to treatment or other medical conditions and is assumed to be related to increased tumor burden, and 4) worsening of tumor-related symptoms deemed clinically significant by physician.

The principles of informed consent described in Food and Drug Administration (FDA) Regulations 21 C.F.R. Part 50 are followed.

The appropriate approvals are obtained from the relevant Institutional Review Board (IRB), the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH), and the Food and Drug Administration (FDA).

While particular embodiments of the invention have been described in detail, it will be apparent to those skilled in the art that these embodiments are exemplary rather than limiting, and the true scope of the invention is that defined within the attached claims.

WHAT IS CLAIMED IS:

1. A process for making a single-vial formulation of polynucleotide/lipid complexes in a pharmaceutically acceptable vehicle for human clinical use *in vivo* or *ex vivo* comprising the steps of:

(a) sterilizing a lipid solution;

(b) sterilizing a polynucleotide solution;

(c) combining the sterilized polynucleotide solution of step (b) with the sterilized lipid solution of step (a), in dilute form, at an ionic strength that is lower than isotonicity, to form polynucleotide/lipid complexes; and

(d) adjusting the polynucleotide/lipid complexes of step (c) to near isotonicity.

2. A process for making a single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use comprising the steps of:

(a) autoclave sterilizing a cationic lipid solution at high concentration;

(b) diluting the sterilized cationic lipid solution of step (a);

(c) filter sterilizing a plasmid DNA solution;

(d) adding the sterilized plasmid DNA solution of step (c) to the diluted sterilized cationic lipid solution of step (b) at an ionic strength that is lower than isotonicity to form DNA/lipid complexes; and

(e) adjusting the DNA/lipid complexes of step (d) to near isotonicity.

3. A process for making a single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use comprising the steps of:

(a) autoclave sterilizing a cationic lipid solution at a concentration sufficiently high to substantially prevent lipid degradation during said autoclave sterilization;

(b) diluting the sterilized cationic lipid solution of step (a) to a degree sufficient to substantially prevent lipid aggregation during step (d) below;

(c) filter sterilizing a plasmid DNA solution;

(d) adding the sterilized plasmid DNA solution of step (c) to the diluted sterilized cationic lipid solution of step (b) at an ionic strength that is lower than isotonicity to form DNA/lipid complexes; and

(e) adjusting the DNA/lipid complexes of step (d) to near isotonicity.

4. A process for making a single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use comprising the steps of:

(a) autoclave sterilizing a cationic lipid solution having a concentration in the range of from about 0.5 to about 5.0 M;

(b) diluting the sterilized cationic lipid solution of step (a) with a diluent to achieve a concentration in the range of from about 0.01 to about 1.0 M;

(c) filter sterilizing a plasmid DNA solution;

(d) adding the sterilized plasmid DNA solution of step (c), having a concentration in the range of from about 0.05 to about 10 mg/mL, to the diluted sterilized cationic lipid solution of step (b) at an ionic strength that is lower than isotonicity to form DNA/lipid complexes; and

(e) adjusting the DNA/lipid complexes of step (d) to near isotonicity.

5. A process for making a single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use comprising the steps of:

(a) autoclave sterilizing a cationic lipid solution of DMRIE/DOPE, having a molar ratio in the range of from about 90:10 to about 10:90, and having a concentration in the range of from about 2 to about 10 mg DMRIE/mL;

(b) diluting the sterilized cationic lipid solution of step (a) with a diluent to achieve a concentration of $\leq$ about 2 mg DMRIE/mL;

(c) filter sterilizing a plasmid DNA solution;

(d) adding the sterilized plasmid DNA solution of step (c), having a concentration of $\leq$ about 10 mg plasmid DNA/mL, to the diluted sterilized cationic lipid solution of step (b) at an ionic strength that is lower than isotonicity to form DNA/lipid complexes at a mass ratio of from about 50:1 to about 1:10 DNA to DMRIE; and

(e) adjusting the DNA/lipid complexes of step (d) to near isotonicity with sodium chloride.

6. A process for making a single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use comprising the steps of:

(a) autoclave sterilizing a cationic lipid solution of DMRIE/DOPE, having a molar ratio of about 50:50, and having a concentration of about 8 mg DMRIE/mL;

(b) diluting the sterilized cationic lipid solution of step (a) with a diluent to achieve a concentration of $\leq$ about 1 mg DMRIE/mL;

(c) filter sterilizing a plasmid DNA solution;

(d) adding the sterilized plasmid DNA solution of step (c), having a concentration of $\leq$ about 5 mg plasmid DNA/mL, to the diluted sterilized cationic lipid solution of step (b) at an ionic strength that is lower than isotonicity to form DNA/lipid complexes at a mass ratio of about 5:1 DNA to DMRIE; and

(e) adjusting the DNA/lipid complexes of step (d) to near physiological salinity.

7. A process for making a single-vial formulation of plasmid DNA/cationic lipid complexes in about 0.9% sodium chloride with about 1% glycerol and about 0.01% Vitamin E for human clinical use comprising the steps of:

(a) autoclave sterilizing a cationic lipid solution of DMRIE/DOPE, having a molar ratio of about 50:50 molar ratio, and having a concentration of about 8 mg DMRIE/mL;

(b) diluting the sterilized cationic lipid solution of step (a) with a diluent to achieve a concentration of $\leq$ about 1 mg DMRIE/mL, and with glycerol and Vitamin E;

(c) filter sterilizing a plasmid DNA solution;

(d) adding the sterilized plasmid DNA solution of step (c), having a concentration of $\leq$ about 5 mg plasmid DNA/mL, to the diluted sterilized cationic lipid solution of step (b) at an ionic strength that is lower than isotonicity to form DNA/lipid complexes at a mass ratio of about 5:1 DNA to DMRIE; and

(e) adjusting the DNA/lipid complexes of step (d) to about 0.9% sodium chloride, wherein the final concentration of glycerol is about 1% and of Vitamin E is about 0.01%.

8. A single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use prepared by the process of Claim 1.

9. A single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use prepared by the process of Claim 2.

10. A single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use prepared by the process of Claim 3.

11. A single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use prepared by the process of Claim 4.

12. A single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use prepared by the process of Claim 5.

13. A single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use prepared by the process of Claim 6.

14. A single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use prepared by the process of Claim 7.

15. A single-vial formulation of plasmid DNA/cationic lipid complexes for human clinical use comprising a cationic lipid component and a plasmid DNA component, wherein said plasmid DNA component and said cationic lipid component are combined at an ionic strength that is lower than isotonicity to form said plasmid DNA/cationic lipid complexes, and further comprising a nearly isotonic aqueous medium, optionally containing no buffering agent except the plasmid DNA itself.

16. The single-vial formulation of Claim 15 that is stable in frozen, refrigerated, room temperature, or body temperature form.

17. The single-vial formulation of Claim 18 that is stable in frozen, refrigerated, or room temperature form for at least about 8 weeks.

18. The single-vial formulation of Claim 15 further comprising about 1% glycerol and about 0.01% Vitamin E in nearly physiological saline.

19. A single-vial formulation for human clinical use comprising plasmid DNA/cationic lipid complexes having storage stability in frozen, refrigerated, or room temperature form for at least about 8 weeks.

20. A single-vial formulation for human clinical use comprising plasmid DNA/cationic lipid complexes retaining *in vitro* transfection efficacy of freshly prepared plasmid DNA/cationic lipid complexes for at least about 8 weeks.

AMENDED CLAIMS

[received by the International Bureau on 2 October 1996 (02.10.96); original claims 1-20 replaced by new claims 1-12 (4 pages)]

1. A process for making a single-vial formulation of polynucleotide/lipid complexes comprising the steps of:

(a) sterilizing a lipid solution diluted with a diluent having an ionic strength that is lower than isotonicity either before or after said sterilization, provided that said diluent is sterile if added after said sterilization;

(b) sterilizing a polynucleotide solution;

(c) combining the sterilized polynucleotide solution of step (b) with the diluted sterilized lipid solution of step (a) to form polynucleotide/lipid complexes; and

(d) adjusting the polynucleotide/lipid complexes of step (c) to near isotonicity.

2. A process for making a single-vial formulation of plasmid DNA/cationic lipid complexes comprising the steps of:

(a) sterilizing a cationic lipid solution at a high concentration;

(b) diluting the sterilized cationic lipid solution of step (a) with a sterile diluent having an ionic strength that is lower than isotonicity;

(c) sterilizing a plasmid DNA solution;

(d) adding the sterilized plasmid DNA solution of step (c) to the diluted sterilized cationic lipid solution of step (b) to form DNA/lipid complexes; and

(e) adjusting the DNA/lipid complexes of step (d) to near isotonicity.

3. A process for making a single-vial formulation of plasmid DNA/cationic lipid complexes comprising the steps of:

(a) sterilizing a cationic lipid solution at a high concentration effective to prevent lipid degradation during said sterilization;

(b) diluting the sterilized cationic lipid solution of step (a) with a sterile diluent having an ionic strength that is lower than isotonicity effective to prevent lipid aggregation during step (d) below;

(c) sterilizing a plasmid DNA solution;

(d) adding the sterilized plasmid DNA solution of step (c) to the diluted

sterilized cationic lipid solution of step (b) to form DNA/lipid complexes; and

(e) adjusting the DNA/lipid complexes of step (d) to near isotonicity.

4. A process for making a single-vial formulation of plasmid DNA/cationic lipid complexes comprising the steps of:

(a) sterilizing a cationic lipid solution having a concentration in the range of from about 0.5 to about 5.0 M;

(b) diluting the sterilized cationic lipid solution of step (a) with a sterile diluent having an ionic strength that is lower than isotonicity to achieve a concentration in the range of from about 0.01 to about 1.0 M;

(c) sterilizing a plasmid DNA solution;

(d) adding the sterilized plasmid DNA solution of step (c), having a concentration in the range of from about 0.05 to about 10 mg/mL, to the diluted sterilized cationic lipid solution of step (b) to form DNA/lipid complexes; and

(e) adjusting the DNA/lipid complexes of step (d) to near isotonicity.

5. A process for making a single-vial formulation of plasmid DNA/cationic lipid complexes comprising the steps of:

(a) sterilizing a cationic lipid solution of DMRIE/DOPE, having a molar ratio in the range of from about 90:10 to about 10:90, and having a concentration in the range of from about 2 to about 10 mg DMRIE/mL;

(b) diluting the sterilized cationic lipid solution of step (a) with a sterile diluent having an ionic strength that is lower than isotonicity to achieve a concentration of $\leq$ about 2 mg DMRIE/mL;

(c) sterilizing a plasmid DNA solution;

(d) adding the sterilized plasmid DNA solution of step (c), having a concentration of $\leq$ about 10 mg plasmid DNA/mL, to the diluted sterilized cationic lipid solution of step (b) to form DNA/lipid complexes at a mass ratio of from about 50:1 to about 1:10 DNA to DMRIE; and

(e) adjusting the DNA/lipid complexes of step (d) to near isotonicity with sodium chloride.

6. A process for making a single-vial formulation of plasmid DNA/cationic lipid complexes comprising the steps of:

(a) sterilizing a cationic lipid solution of DMRIE/DOPE, having a molar ratio of about 50:50, and having a concentration of about 8 mg DMRIE/mL;

(b) diluting the sterilized cationic lipid solution of step (a) with a sterile diluent having an ionic strength that is lower than isotonicity to achieve a concentration of ≤ about 1 mg DMRIE/mL;

(c) sterilizing a plasmid DNA solution;

(d) adding the sterilized plasmid DNA solution of step (c), having a concentration of ≤ about 5 mg plasmid DNA/mL, to the diluted sterilized cationic lipid solution of step (b) to form DNA/lipid complexes at a mass ratio of about 5:1 DNA to DMRIE; and

(e) adjusting the DNA/lipid complexes of step (d) to near physiological salinity.

7. A process for making a single-vial formulation of plasmid DNA/cationic lipid complexes in about 0.9% sodium chloride with about 1% glycerol and about 0.01% Vitamin E comprising the steps of:

(a) sterilizing a cationic lipid solution of DMRIE/DOPE, having a molar ratio of about 50:50 molar ratio, and having a concentration of about 8 mg DMRIE/mL;

(b) diluting the sterilized cationic lipid solution of step (a) with a sterile diluent having an ionic strength that is lower than isotonicity to achieve a concentration of ≤ about 1 mg DMRIE/mL, and with glycerol and Vitamin E;

(c) sterilizing a plasmid DNA solution;

(d) adding the sterilized plasmid DNA solution of step (c), having a concentration of ≤ about 5 mg plasmid DNA/mL, to the diluted sterilized cationic lipid solution of step (b) to form DNA/lipid complexes at a mass ratio of about 5:1 DNA to DMRIE; and

(e) adjusting the DNA/lipid complexes of step (d) to about 0.9% sodium chloride, wherein the final concentration of glycerol is about 1% and of Vitamin E is about 0.01%.

8. The single-vial formulation of any of Claims 1-7 that is stable in frozen, refrigerated, room temperature, or body temperature form.

9. The single-vial formulation of any of Claims 1-7 that is stable in frozen, refrigerated, or room temperature form for at least about 8 weeks.

10. The single-vial formulation of any of Claims 1-7 that retains *in vitro* transfection efficacy of freshly prepared plasmid DNA/cationic lipid complexes for at least about 8 weeks.

11. The single-vial formulation of any of Claims 1-7, wherein said diluent is water.

12. The single-vial formulation of any of Claims 1-7, wherein said polynucleotide solution or said plasmid DNA solution contains water as the solvent.

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCI/US 96/05035

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/88 A61K9/127 A61K47/48 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 16024 (VICAL INC) 31 October 1991 see page 7, line 25 - page 14, line 36 see page 33, line 32 - page 37, line 25 see page 51 - page 56; examples 13-17 ---	1-20
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 4, 28 January 1994, pages 2550-2561, XP002010485 FELGNER ET AL: "ENHANCED GENE DELIVERY AND MECHANISM STUDIES WITH A NOVEL SERIES OF CATIONIC LIPID FORMULATIONS" cited in the application	15-20
A	see page 2550,abstract;pages 2551-2552,'experimental procedures' see figure 2 ---	1-14
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
8 August 1996	19.08.96

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